METHODS FOR PRODUCING A POLYPEPTIDE IN A BACILLUS CELL

Cross-Reference to Related Applications

This application is a continuation-in-part of application serial no. 09/031,442 filed February 26, 1998, the contents of which are fully incorporated herein by reference.

Background of the Invention

Field of the Invention

The present invention relates to methods for producing a polypeptide in a bacterial cell.

Description of the Related Art

Bacilli are well established as host cell systems for the production of native and recombinant proteins. However, *Bacillus* host cells with the desirable traits of increased protein expression may not necessarily have the most desirable characteristics for commercial use.

Conventionally, maximal expression of a gene contained in a *Bacillus* cell is achieved by amplifying in the chromosome an expression cassette containing a single promoter operably linked to a gene of interest and an amplifiable selectable marker gene, *e.g.*, an antibiotic resistance marker. The amplification leads to the production of multiple copies of the expression cassette and the selectable marker gene in the chromosome.

However, there are disadvantages associated with this approach. For example, it may not be possible to achieve saturating levels of mRNA by amplifying genes driven by single promoters. Also, the production of multiple copies of the expression cassette and the selectable marker gene in the chromosome of a cell may not be stable. Furthermore, the removal of the selectable marker genes without also losing the expression cassettes may not be technically feasible.

Ichikawa *et al.* (1993, *FEMS Microbiological Letters* 111: 219-224) disclose the extracellular production of cholera toxin B in *Bacillus brevis* containing an expression-

secretion vector with multiple promoters which mediate the expression of the gene encoding the mature cholera toxin B.

Agaisse and Lereclus (1994, *Molecular Microbiology* 13: 97-107) disclose a structural and functional analysis of the promoter region involved in the full expression of the *cryIIIA* toxin gene of *Bacillus thuringiensis*. WO 94/25612 discloses an mRNA stabilizer region downstream of the promoter and upstream of the coding sequence of the *cryIIIA* gene which increases expression of the gene.

Hue *et al.* (1995, *Journal of Bacteriology* 177: 3465-3471) disclose a 5' mRNA stabilizer sequence which stabilized several heterologous RNA sequences when present at the 5' end and increased expression of downstream coding sequences several-fold in *Bacillus subtilis*.

It is an object of the present invention to provide improved methods for producing a polypeptide in a *Bacillus* strain.

Summary of the Invention

The present invention relates to methods for producing a polypeptide, comprising: (a) cultivating a *Bacillus* host cell in a medium conducive for the production of the polypeptide, wherein the *Bacillus* cell comprises a nucleic acid construct comprising (i) a tandem promoter in which each promoter sequence of the tandem promoter is operably linked to a nucleic acid sequence encoding the polypeptide and alternatively also (ii) an mRNA processing/stabilizing sequence located downstream of the tandem promoter and upstream of the nucleic acid sequence encoding the polypeptide; and (b) isolating the polypeptide from the cultivation medium.

The present invention also relates to *Bacillus* cells comprising a nucleic acid construct which comprises (i) a tandem promoter in which each promoter sequence of the tandem promoter is operably linked to a single copy of a nucleic acid sequence encoding a polypeptide and alternatively also (ii) an mRNA processing/stabilizing sequence located downstream of the tandem promoter and upstream of the nucleic acid sequence encoding the polypeptide.

The present invention also relates to methods for producing a polypeptide, comprising: (a) cultivating a *Bacillus* host cell in a medium conducive for the production of

the polypeptide, wherein the *Bacillus* cell comprises a nucleic acid construct comprising (i) a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region operably linked to a single copy of a nucleic acid sequence encoding the polypeptide and (ii) an mRNA processing/stabilizing sequence located downstream of the "consensus" promoter and upstream of the nucleic acid sequence encoding the polypeptide; and (b) isolating the polypeptide from the cultivation medium.

The present invention also relates to *Bacillus* cells comprising a nucleic acid construct which comprises (i) a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region operably linked to a single copy of a nucleic acid sequence encoding a polypeptide and (ii) an mRNA processing/stabilizing sequence located downstream of the "consensus" promoter and upstream of the nucleic acid sequence encoding the polypeptide.

The present invention also relates to methods for producing a polypeptide, comprising: (a) cultivating a *Bacillus* cell in a medium conducive for the production of the polypeptide, wherein the *Bacillus* cell comprises a nucleic acid sequence encoding the polypeptide operably linked to a "consensus" *amyQ* promoter; and (b) isolating the polypeptide from the cultivation medium.

The present invention also relates to *Bacillus* cells comprising a nucleic acid construct which comprises (i) a "consensus" *amyQ* promoter operably linked to a single copy of a nucleic acid sequence encoding a polypeptide and (ii) an mRNA processing/stabilizing sequence located downstream of the "consensus" *amyQ* promoter and upstream of the nucleic acid sequence encoding the polypeptide.

The present invention also relates to methods for producing selectable marker-free mutants of a *Bacillus* cell.

Brief Description of the Figures

Figure 1 shows a map of the *cryIIIA* promoter and the *cryIIIA* processing/stabilizing sequence.

Figure 2 shows schematically various constructions containing a tandem promoter and the *cryIIIA* mRNA processing/stabilizing sequence.

Figure 3 shows a restriction map of pDG268MCS.

Figure 4 shows a restriction map of pDG268MCSΔ-Pr$_{cryIIIA}$/cryIIIAstab/SAV.

Figure 5 shows a restriction map of pDG268MCSΔneo-Pr$_{cryIIIA}$/cryIIIAstab/SAV.

Figure 6 shows a restriction map of pHP13amp-MCS.

Figure 7 shows a restriction map of pSX222.

Figure 8 shows a restriction map of pHP13amp-SAV.

Figure 9 shows a restriction map of pDG268MCS-Pr$_{amyL}$/SAV.

Figure 10 shows a restriction map of pDG268MCS-Pr$_{amyQ}$/SAV.

Figure 11 shows a restriction map of pUC18-Pr$_{cryIIIA}$/cryIIIAstab/cryIIIA.

Figure 12 shows a restriction map of pDG268MCS-Pr$_{cryIIIA}$/cryIIIAstab/SAV.

Figure 13 shows a restriction map of pDG268MCSΔneo-Pr$_{cryIIIA}$/SAV.

Figure 14 shows a restriction map of pDG268MCS-Pr$_{amyL}$/cryIIIAstab/SAV.

Figure 15 shows a restriction map of pDG268MCSΔneo-Pr$_{amyL}$/Pr$_{cryIIIA}$/cryIIIAstab/SAV.

Figure 16 shows a restriction map of pDG268MCSΔneo-Pr$_{amyL}$/Pr$_{cryIIIA}$/SAV.

Figure 17 shows a restriction map of pDG268MCSΔneo-Pr$_{amyQ}$/cryIIIAstab/SAV.

Figure 18 shows a restriction map of pDG268MCS-Pr$_{amyQ}$/Pr$_{cryIIIA}$/cryIIIAstab/SAV.

Figure 19 shows a restriction map of pDG268MCSΔneo-Pr$_{amyQ}$/Pr$_{cryIIIA}$/SAV.

Figure 20 shows a restriction map of pDG268MCSΔneo-long cryIIIAstab/SAV.

Figure 21 shows a nucleic acid sequence containing the "consensus" amyQ promoter.

Figure 22 shows a restriction map of pDG268MCS-Pr$_{short\ "consensus"\ amyQ}$/SAV.

Figure 23 shows a restriction map of pDG268MCS-Pr$_{short\ "consensus"\ amyQ\ dimer}$/SAV.

Figure 24 shows a restriction map of pDG268MCSΔ-Pr$_{short\ "consensus"\ amyQ}$/cryIIIAstab/SAV.

Figure 25 shows a restriction map of pDG268MCSΔneo-Pr$_{short\ "consensus"\ amyQ}$/Pr$_{cryIIIA}$/cryIIIAstab/SAV.

Figure 26 shows a restriction map of pDG268MCSΔneo-Pr$_{short\ "consensus"\ amyQ}$/long cryIIIAstab/SAV.

Figure 27 shows a restriction map of pDG268MCS-Pr$_{"short"\ amyQ}$/SAV.

Figure 28 shows a restriction map of pDG268Δneo-Pr$_{"short"\ amyQ\ dimer}$/SAV.

Figure 29 shows a restriction map of pDG268Δneo-Pr$_{"short"\ amyQ\ dimer}$/cryIIIAstab/SAV.

Detailed Description of the Invention

In a first embodiment, the present invention relates to methods for producing a polypeptide, comprising: (a) cultivating a *Bacillus* cell in a medium conducive for the production of the polypeptide, wherein the *Bacillus* cell comprises a nucleic acid construct comprising (i) a tandem promoter in which each promoter sequence of the tandem promoter is operably linked to a single copy of a nucleic acid sequence encoding the polypeptide and alternatively also (ii) an mRNA processing/stabilizing sequence located downstream of the tandem promoter and upstream of the nucleic acid sequence encoding the polypeptide; and (b) isolating the polypeptide from the cultivation medium.

In a second embodiment, the present invention relates to methods for producing a polypeptide, comprising: (a) cultivating a *Bacillus* host cell in a medium conducive for the production of the polypeptide, wherein the *Bacillus* cell comprises a nucleic acid construct comprising (i) a "consensus" promoter operably linked to a single copy of a nucleic acid sequence encoding the polypeptide and (ii) an mRNA processing/stabilizing sequence located downstream of the "consensus" promoter and upstream of the nucleic acid sequence encoding the polypeptide; and (b) isolating the polypeptide from the cultivation medium.

In a third embodiment, the present invention relates to methods for producing a polypeptide, comprising: (a) cultivating a *Bacillus* cell in a medium conducive for the production of the polypeptide, wherein the *Bacillus* cell comprises a nucleic acid sequence encoding the polypeptide operably linked to a "consensus" *amyQ* promoter; and (b) isolating the polypeptide from the cultivation medium.

"Promoter" is defined herein as a nucleic acid sequence involved in the binding of RNA polymerase to initiate transcription of a gene. "Tandem promoter" is defined herein as two or more promoter sequences each of which is operably linked to a coding sequence and mediates the transcription of the coding sequence into mRNA. "Operably linked" is defined herein as a configuration in which a control sequence, *e.g.*, a promoter sequence, is appropriately placed at a position relative to a coding sequence such that the control sequence directs the production of a polypeptide encoded by the coding sequence. "Coding sequence" is defined herein as a nucleic acid sequence which is transcribed into mRNA and translated into a polypeptide when placed under the control of the appropriate control

sequences. The boundaries of the coding sequence are generally determined by a ribosome binding site located just upstream of the open reading frame at the 5' end of the mRNA and a transcription terminator sequence located just downstream of the open reading frame at the 3' end of the mRNA. A coding sequence can include, but is not limited to, genomic DNA, cDNA, semisynthetic, synthetic, and recombinant nucleic acid sequences. "Nucleic acid construct" is defined herein as a nucleic acid molecule, either single- or double-stranded, which is isolated from a naturally occurring gene or which has been modified to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature. The term nucleic acid construct is synonymous with the term expression cassette when the nucleic acid construct contains all the control sequences required for expression of a coding sequence.

Each promoter sequence of the tandem promoter may be any nucleic acid sequence which shows transcriptional activity in the *Bacillus* cell of choice including a mutant, truncated, and hybrid promoter, and may be obtained from genes encoding extracellular or intracellular polypeptides either homologous or heterologous to the *Bacillus* cell. Each promoter sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide and native or foreign to the *Bacillus* cell. The promoter sequences may be the same promoter sequence or different promoter sequences.

In a preferred embodiment, the promoter sequences may be obtained from a bacterial source. In a more preferred embodiment, the promoter sequences may be obtained from a gram positive bacterium such as a *Bacillus* strain, *e.g., Bacillus alkalophilus, Bacillus amyloliquefaciens, Bacillus brevis, Bacillus circulans, Bacillus clausii, Bacillus coagulans, Bacillus firmus, Bacillus lautus, Bacillus lentus, Bacillus licheniformis, Bacillus megaterium, Bacillus pumilus, Bacillus stearothermophilus, Bacillus subtilis*, or *Bacillus thuringiensis*; or a *Streptomyces* strain, *e.g., Streptomyces lividans* or *Streptomyces murinus*; or from a gram negative bacterium, *e.g., E. coli* or *Pseudomonas* sp.

An example of a suitable promoter for directing the transcription of a nucleic acid sequence in the methods of the present invention is the promoter obtained from the *E. coli lac* operon. Another example is the promoter of the *Streptomyces coelicolor* agarase gene (*dagA*). Another example is the promoter of the *Bacillus lentus* alkaline protease gene (*aprH*). Another example is the promoter of the *Bacillus licheniformis* alkaline protease

gene (subtilisin Carlsberg gene). Another example is the promoter of the *Bacillus subtilis* levansucrase gene (*sacB*). Another example is the promoter of the *Bacillus* subtilis alpha-amylase gene (*amyE*). Another example is the promoter of the *Bacillus licheniformis* alpha-amylase gene (*amyL*). Another example is the promoter of the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*). Another example is the promoter of the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*). Another example is a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region. Another example is the promoter of the *Bacillus licheniformis* penicillinase gene (*penP*). Another example are the promoters of the *Bacillus subtilis xylA* and *xylB* genes. Another example is the promoter of the *Bacillus thuringiensis* subsp. *tenebrionis* CryIIIA gene (*cryIIIA*, SEQ ID NO. 21) or portions thereof. Another example is the promoter of the prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75:3727-3731). Another example is the promoter of the *spo1* bacterial phage promoter. Another example is the tac promoter (DeBoer *et al.*, 1983, *Proceedings of the National Academy of Sciences USA* 80:21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; and in Sambrook, Fritsch, and Maniatus, 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York.

The two or more promoter sequences of the tandem promoter may simultaneously promote the transcription of the nucleic acid sequence. Alternatively, one or more of the promoter sequences of the tandem promoter may promote the transcription of the nucleic acid sequence at different stages of growth of the *Bacillus* cell.

In a preferred embodiment, the tandem promoter contains at least the *amyQ* promoter of the *Bacillus amyloliquefaciens* alpha-amylase gene. In another preferred embodiment, the tandem promoter contains at least a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region. In another preferred embodiment, the tandem promoter contains at least the *amyL* promoter of the *Bacillus licheniformis* alpha-amylase gene. In another preferred embodiment, the tandem promoter contains at least the *cryIIIA* promoter or portions thereof (Agaisse and Lereclus, 1994, *supra*).

In a more preferred embodiment, the tandem promoter contains at least the *amyL* promoter and the *cryIIIA* promoter. In another more preferred embodiment, the tandem

promoter contains at least the *amyQ* promoter and the *cryIIIA* promoter. In another more preferred embodiment, the tandem promoter contains at least a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region and the *cryIIIA* promoter. In another more preferred embodiment, the tandem promoter contains at least two copies of the *amyL* promoter. In another more preferred embodiment, the tandem promoter contains at least two copies of the *amyQ* promoter. In another more preferred embodiment, the tandem promoter contains at least two copies of a "consensus" promoter having the sequence TTGACA for the "-35" region and TATAAT for the "-10" region. In another more preferred embodiment, the tandem promoter contains at least two copies of the *cryIIIA* promoter.

The construction of a "consensus" promoter may be accomplished by site-directed mutagenesis to create a promoter which conforms more perfectly to the established consensus sequences for the "-10" and "-35" regions of the vegetative "sigma A-type" promoters for *Bacillus subtilis* (Voskuil *et al.*, 1995, *Molecular Microbiology* 17: 271-279). The consensus sequence for the "-35" region is TTGACA and for the "-10" region is TATAAT. The consensus promoter may be obtained from any promoter which can function in a *Bacillus* host cell.

In a preferred embodiment, the "consensus" promoter is obtained from a promoter obtained from the *E. coli lac* operon, *Streptomyces coelicolor* agarase gene (*dagA*), *Bacillus lentus* alkaline protease gene (*aprH*), *Bacillus licheniformis* alkaline protease gene (subtilisin Carlsberg gene), *Bacillus subtilis* levansucrase gene (*sacB*), *Bacillus* subtilis alpha-amylase gene (*amyE*), *Bacillus licheniformis* alpha-amylase gene (*amyL*), *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), *Bacillus licheniformis* penicillinase gene (*penP*), *Bacillus subtilis* xylA and *xylB* genes, *Bacillus thuringiensis* subsp. *tenebrionis* CryIIIA gene (*cryIIIA*, SEQ ID NO. 21) or portions thereof, or prokaryotic beta-lactamase gene *spo1* bacterial phage promoter.

In a more preferred embodiment, the "consensus" promoter is obtained from *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*). In a most preferred embodiment, the consensus promoter is the "consensus" *amyQ* promoter contained in nucleotides 1 to 185 of SEQ ID NO. 26 or SEQ ID NO. 27. In another most preferred embodiment, the consensus promoter is the short "consensus" *amyQ* promoter contained in nucleotides 86 to

185 of SEQ ID NO. 26 or SEQ ID NO. 27. The "consensus" *amyQ* promoter of SEQ ID NO. 26 contains the following mutations of the nucleic acid sequence containing the wild-type *amyQ* promoter (SEQ ID NO. 25): T to A and T to C in the -35 region (with respect to the transcription start site) at positions 135 and 136, respectively, and an A to T change in the -10 region at position 156 of SEQ ID NO. 25. The "consensus" *amyQ* promoter (SEQ ID NO. 27) further contains a T to A change at position 116 approximately 20 base pairs upstream of the -35 region as shown in Figure 21 (SEQ ID NO. 27). This change apparently had no detrimental effect on promoter function since it is well removed from the critical -10 and -35 regions.

"An mRNA processing/stabilizing sequence" is defined herein as a sequence located downstream of one or more promoter sequences and upstream of a coding sequence to which each of the one or more promoter sequences are operably linked such that all mRNAs synthesized from each promoter sequence may be processed to generate mRNA transcripts with a stabilizer sequence at the 5' end of the transcripts. The presence of such a stabilizer sequence at the 5' end of the mRNA transcripts increases their half-life (Agaisse and Lereclus, 1994, *supra*, Hue *et al.*, 1995, *supra*). The mRNA processing/stabilizing sequence is complementary to the 3' extremity of a bacterial 16S ribosomal RNA. In a preferred embodiment, the mRNA processing/stabilizing sequence generates essentially single-size transcripts with a stabilizing sequence at the 5' end of the transcripts.

In a more preferred embodiment, the mRNA processing/stabilizing sequence is the *Bacillus thuringiensis cryIIIA* mRNA processing/stabilizing sequence disclosed in WO 94/25612 and Agaisse and Lereclus, 1994, *supra*, or portions thereof which retain the mRNA processing/stabilizing function. In another more preferred embodiment, the mRNA processing/stabilizing sequence is the *Bacillus subtilis* SP82 mRNA processing/stabilizing sequence disclosed in Hue *et al.*, 1995, *supra*, or portions thereof which retain the mRNA processing/stabilizing function.

When the *cryIIIA* promoter and its mRNA processing/stabilizing sequence are employed in the methods of the present invention, a DNA fragment containing the sequence disclosed in WO 94/25612 and Agaisse and Lereclus, 1994, *supra*, delineated by nucleotides -635 to -22 (Figure 1) (SEQ ID NO. 21), or portions thereof which retain the promoter and mRNA processing/stabilizing functions, may be used. The *cryIIIA* promoter is delineated by nucleotides -635 to -552 while the *cryIIIA* mRNA processing/stabilizing

sequence is contained within nucleotides -551 to -22. In a preferred embodiment, the *cryIIIA* mRNA processing/stabilizing sequence is contained in a fragment comprising nucleotides -568 to -22. In another preferred embodiment, the *cryIIIA* mRNA processing/stabilizing sequence is contained in a fragment comprising nucleotides -367 to -21. Furthermore, DNA fragments containing only the *cryIIIA* promoter or only the *cryIIIA* mRNA processing/stabilizing sequence may be prepared using methods well known in the art to construct various tandem promoter and mRNA processing/stabilizing sequence combinations. In this embodiment, the *cryIIIA* promoter and its mRNA processing/stabilizing sequence are preferably placed downstream of the other promoter sequence(s) constituting the tandem promoter and upstream of the coding sequence of the gene of interest. Various constructions containing a tandem promoter and the *cryIIIA* mRNA processing/stabilizing sequence are shown in Figure 2.

The *Bacillus* cell may contain one or more copies of the nucleic acid construct. In a preferred embodiment, the *Bacillus* cell contains a single copy of the nucleic acid construct.

The nucleic acid construct may further contain one or more selectable markers which permit easy selection of transformed cells. A selectable marker is a gene the product of which provides for biocide resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Examples of bacterial selectable markers are the *dal* genes from *Bacillus subtilis* or *Bacillus licheniformis*, or markers which confer antibiotic resistance such as ampicillin, kanamycin, erythromycin, chloramphenicol or tetracycline resistance. Furthermore, selection may be accomplished by co-transformation, *e.g.*, as described in WO 91/09129, where the selectable marker is on a separate vector.

A particular advantage of the present invention is that a *Bacillus* cell can be produced free of a selectable marker gene, *i.e.*, after the introduction of the nucleic acid construct into the *Bacillus* cell, the selectable marker gene can be deleted from the *Bacillus* cell making the cell marker-free. The nucleic acid construct containing a single copy of the nucleic acid sequence encoding the polypeptide allows for the removal of the selectable marker gene to produce a *Bacillus* cell free of such a marker which may be preferable for regulatory and environmental reasons.

Gene deletion or replacement techniques may be used for the complete removal of the selectable marker gene. In such methods, the deletion of the selectable marker gene may be accomplished by homologous recombination using a plasmid which has been

constructed to contiguously contain the 5' and 3' regions flanking the selectable marker gene. The contiguous 5' and 3' regions may be introduced into a *Bacillus* cell on a temperature-sensitive plasmid, *e.g.*, pE194, in association with a second selectable marker at a permissive temperature to allow the plasmid to become established in the cell. The cell is then shifted to a non-permissive temperature to select for cells which have the plasmid integrated into the chromosome at one of the homologous flanking regions. Selection for integration of the plasmid is effected by selection for the second selectable marker. After integration, a recombination event at the second homologous flanking region is stimulated by shifting the cells to the permissive temperature for several generations without selection. The cells are plated to obtain single colonies and the colonies are examined for loss of both selectable markers (see, for example, Perego, 1993, *In* A.L. Sonneshein, J.A. Hoch, and R. Losick, editors, *Bacillus subtilis and Other Gram-Positive Bacteria*, Chapter 42, American Society of Microbiology, Washington, D.C., 1993).

The polypeptide may be native or heterologous to the *Bacillus* cell. The term "polypeptide" is not meant herein to refer to a specific length of the encoded product and, therefore, encompasses peptides, oligopeptides, and proteins. The polypeptide may also be a naturally occurring allelic or engineered variant of a polypeptide.

Preferably, the polypeptide is a hormone or variant thereof, enzyme, receptor or portion thereof, antibody or portion thereof, or reporter. In a more preferred embodiment, the polypeptide is an oxidoreductase. In another more preferred embodiment, the polypeptide is a transferase. In another more preferred embodiment, the polypeptide is a hydrolase. In another more preferred embodiment, the polypeptide is a lyase. In another more preferred embodiment, the polypeptide is an isomerase. In another more preferred embodiment, the polypeptide is a ligase.

In an even more preferred embodiment, the polypeptide is an aminopeptidase. In another even more preferred embodiment, the polypeptide is an amylase. In another even more preferred embodiment, the polypeptide is a carbohydrase. In another even more preferred embodiment, the polypeptide is a carboxypeptidase. In another even more preferred embodiment, the polypeptide is a catalase. In another even more preferred embodiment, the polypeptide is a cellulase. In another even more preferred embodiment, the polypeptide is a chitinase. In another even more preferred embodiment, the polypeptide is a cutinase. In another even more preferred embodiment, the polypeptide is a

cyclodextrin glycosyltransferase. In another even more preferred embodiment, the polypeptide is a deoxyribonuclease. In another even more preferred embodiment, the polypeptide is an esterase. In another even more preferred embodiment, the polypeptide is an alpha-galactosidase. In another even more preferred embodiment, the polypeptide is a beta-galactosidase. In another even more preferred embodiment, the polypeptide is a glucoamylase. In another even more preferred embodiment, the polypeptide is an alpha-glucosidase. In another even more preferred embodiment, the polypeptide is a beta-glucosidase. In another even more preferred embodiment, the polypeptide is an invertase. In another even more preferred embodiment, the polypeptide is a laccase. In another even more preferred embodiment, the polypeptide is a lipase. In another even more preferred embodiment, the polypeptide is a mannosidase. In another even more preferred embodiment, the polypeptide is a mutanase. In another even more preferred embodiment, the polypeptide is an oxidase. In another even more preferred embodiment, the polypeptide is a pectinolytic enzyme. In another even more preferred embodiment, the polypeptide is a peroxidase. In another even more preferred embodiment, the polypeptide is a phytase. In another even more preferred embodiment, the polypeptide is a polyphenoloxidase. In another even more preferred embodiment, the polypeptide is a proteolytic enzyme. In another even more preferred embodiment, the polypeptide is a ribonuclease. In another even more preferred embodiment, the polypeptide is a transglutaminase. In another even more preferred embodiment, the polypeptide is a xylanase.

In a most preferred embodiment, the polypeptide is a serine protease, for example, a subtilisin. In another most preferred embodiment, the polypeptide is a maltogenic amylase. In another most preferred embodiment, the polypeptide is a pullulanase.

The methods of the present invention may also be used for the recombinant production of polypeptides which are native to the *Bacillus* cell. The present invention also encompasses, within the scope of the term "heterologous polypeptide", such recombinant production of native polypeptides, to the extent that such expression involves the use of genetic elements not native to the *Bacillus* cell, or use of native elements which have been manipulated to function in a manner not normally occurring in the host cell.

In the methods of the present invention, heterologous polypeptides may also include fused or hybrid polypeptides in which another polypeptide is fused at the N-terminus or the C-terminus of the polypeptide or fragment thereof. A fused polypeptide is produced by

fusing a nucleic acid sequence (or a portion thereof) encoding one polypeptide to a nucleic acid sequence (or a portion thereof) encoding another polypeptide. Techniques for producing fusion polypeptides are known in the art, and include ligating the coding sequences encoding the polypeptides so that they are in frame and expression of the fused polypeptide is under control of the same promoter(s) and terminator. The hybrid polypeptides may comprise a combination of partial or complete polypeptide sequences obtained from at least two different polypeptides wherein one or more may be heterologous to the *Bacillus* cell.

The construction of a *Bacillus* cell comprising a tandem or "consensus" promoter and alternatively also an mRNA processing/stabilizing sequence, operably linked to a nucleic acid sequence encoding a polypeptide of interest may be accomplished by modifying an isolated nucleic acid sequence encoding the polypeptide using methods well known in the art to operably link the promoter and alternatively also the mRNA processing/stabilizing sequence to the nucleic acid sequence, inserting the modified sequence into a vector, and introducing the vector into the *Bacillus* cell's chromosome by homologous recombination or into the *Bacillus* cell as an extrachromosomal autonomously replicating element, *e.g.*, plasmid. However, it will be understood that the nucleic acid sequence may also be manipulated *in vivo* in the *Bacillus* cell using methods well known in the art.

In the methods of the present invention, a nucleic acid sequence encoding a polypeptide of interest may be native or foreign to the *Bacillus* cell. A foreign nucleic acid sequence encoding a heterologous polypeptide may be obtained from any prokaryotic, eukaryotic, or other source, *e.g.*, archaebacteria. In a preferred embodiment, the nucleic acid sequence is obtained from a bacterial strain, such as a gram-negative or a gram-positive bacterial cell. In a more preferred embodiment, the nucleic acid sequence encoding the polypeptide is obtained from a *Bacillus* cell. For purposes of the present invention, the term "obtained from" as used herein in connection with a given source shall mean that the polypeptide is produced by the source or by a cell in which a gene from the source has been inserted.

The techniques used to isolate or clone a nucleic acid sequence encoding a polypeptide are well known in the art and include, for example, isolation from genomic DNA, preparation from cDNA, or a combination thereof. The cloning of the nucleic acid sequences from such genomic DNA can be effected, *e.g.*, by using antibody screening of

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expression libraries to detect cloned DNA fragments with shared structural features or the well known polymerase chain reaction (PCR). See, for example, Innis *et al.*, 1990, *PCR Protocols: A Guide to Methods and Application*, Academic Press, New York. Other nucleic acid amplification procedures such as ligase chain reaction (LCR), ligated activated transcription (LAT), and nucleic acid sequence-based amplification (NASBA) may be used. The cloning procedures may involve excision and isolation of a desired nucleic acid fragment comprising the nucleic acid sequence encoding the polypeptide, insertion of the fragment into a vector molecule, and incorporation of the recombinant vector into a *Bacillus* cell where clones of the nucleic acid sequence will be replicated. The nucleic acid sequence may be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combinations thereof.

An isolated nucleic acid sequence encoding a polypeptide of interest may then be manipulated to produce a nucleic acid construct by operably linking the nucleic acid sequence to a tandem or "consensus" promoter and alternatively also to an mRNA processing/stabilizing sequence, as well as one or more additional control sequences which direct the expression of the coding sequence in a *Bacillus* cell under conditions compatible with the control sequences. Expression will be understood to include any step involved in the production of the polypeptide including, but not limited to, transcription, post-transcriptional modification, translation, post-translational modification, and secretion. The techniques for modifying nucleic acid sequences utilizing cloning methods are well known in the art.

The term "control sequences" is defined herein to include all components which are necessary or advantageous for expression of the coding sequence of the nucleic acid sequence. Each control sequence may be native or foreign to the nucleic acid sequence encoding the polypeptide. In addition to the tandem or "consensus" promoter, described earlier, such control sequences include, but are not limited to, a leader, a signal sequence, and a transcription terminator. The control sequences may be provided with linkers for the purpose of introducing specific restriction sites facilitating ligation of the control sequences with the coding region of the nucleic acid sequence encoding a polypeptide.

The control sequence may also be a suitable transcription terminator sequence, a sequence recognized by a *Bacillus* cell to terminate transcription. The terminator sequence is operably linked to the 3' terminus of the nucleic acid sequence encoding the polypeptide.

Any terminator which is functional in the *Bacillus* cell of choice may be used in the present invention.

The control sequence may also be a suitable leader sequence, a nontranslated region of an mRNA which is important for translation by the *Bacillus* cell. The leader sequence is operably linked to the 5' terminus of the nucleic acid sequence encoding the polypeptide. Any leader sequence which is functional in the *Bacillus* cell of choice may be used in the present invention.

The control sequence may also be a signal peptide coding region that codes for an amino acid sequence linked to the amino terminus of the polypeptide which directs the expressed polypeptide into the cell's secretory pathway. The signal peptide coding region may be native to the polypeptide or may be obtained from foreign sources. The 5' end of the coding sequence of the nucleic acid sequence may inherently contain a signal peptide coding region naturally linked in translation reading frame with the segment of the coding region which encodes the secreted polypeptide. Alternatively, the 5' end of the coding sequence may contain a signal peptide coding region which is foreign to that portion of the coding sequence which encodes the secreted polypeptide. The foreign signal peptide coding region may be required where the coding sequence does not normally contain a signal peptide coding region. Alternatively, the foreign signal peptide coding region may simply replace the natural signal peptide coding region in order to obtain enhanced secretion of the polypeptide relative to the natural signal peptide coding region normally associated with the coding sequence. The signal peptide coding region may be obtained from an amylase or a protease gene from a *Bacillus* species. However, any signal peptide coding region capable of directing the expressed polypeptide into the secretory pathway of a *Bacillus* cell of choice may be used in the present invention.

An effective signal peptide coding region for *Bacillus* cells is the signal peptide coding region obtained from the *Bacillus* NCIB 11837 maltogenic amylase gene, *Bacillus stearothermophilus* alpha-amylase gene, *Bacillus licheniformis* subtilisin gene, *Bacillus licheniformis* beta-lactamase gene, *Bacillus stearothermophilus* neutral protease genes (*nprT*, *nprS*, *nprM*), and *Bacillus subtilis prsA* gene. Further signal peptides are described by Simonen and Palva, 1993, *Microbiological Reviews* 57: 109-137.

In the methods of the present invention, a recombinant expression vector comprising a nucleic acid sequence encoding a polypeptide of interest, a tandem or "consensus"

promoter and alternatively also an mRNA processing/stabilizing sequence, and transcriptional and translational stop signals may be used for the recombinant production of a polypeptide. The various nucleic acid and control sequences described above may be joined together to produce a recombinant expression vector which may include one or more convenient restriction sites to allow for insertion or substitution of the nucleic acid sequence encoding the polypeptide at such sites. Alternatively, the nucleic acid sequence may be expressed by inserting the nucleic acid sequence or a nucleic acid construct comprising the sequence into an appropriate vector for expression. In creating the expression vector, the coding sequence is located in the vector so that the coding sequence is operably linked with a tandem or "consensus" promoter and alternatively also an mRNA processing/stabilizing sequence, and any other appropriate control sequences for expression, and possibly secretion.

The recombinant expression vector may be any vector which can be conveniently subjected to recombinant DNA procedures and can bring about the expression of the nucleic acid sequence on introduction into a *Bacillus* cell. The choice of the vector will typically depend on the compatibility of the vector with the *Bacillus* cell into which the vector is to be introduced. The vectors may be linear or closed circular plasmids. The vector may be an autonomously replicating vector, *i.e.*, a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, *e.g.*, a plasmid, an extrachromosomal element, a minichromosome, or an artificial chromosome. The vector may contain any means for assuring self-replication. Alternatively, the vector may be one which, when introduced into the *Bacillus* cell, is integrated into the genome and replicated together with the chromosome(s) into which it has been integrated. The vector system may be a single vector or plasmid or two or more vectors or plasmids, or a transposon.

"Introduction" means introducing a vector comprising the nucleic acid sequence into a *Bacillus* cell so that the vector is maintained as a chromosomal integrant or as a self-replicating extrachromosomal vector. Integration is generally considered to be an advantage as the nucleic acid sequence is more likely to be stably maintained in the cell. Integration of the vector into the chromosome occurs by homologous recombination, non-homologous recombination, or transposition.

The introduction of an expression vector into a *Bacillus* cell may, for instance, be effected by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, *e.g.*, Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5271-5278).

For integration, the vector may rely on the nucleic acid sequence encoding the polypeptide or any other element of the vector for stable integration of the vector into the genome by homologous recombination. The vector may contain additional nucleic acid sequences for directing integration by homologous recombination into the genome of the *Bacillus* cell. The additional nucleic acid sequences enable the vector to be integrated into the *Bacillus* cell genome at a precise location in the chromosome. To increase the likelihood of integration at a precise location, the integrational elements should preferably contain a sufficient number of nucleic acids, such as 100 to 1,500 base pairs, preferably 400 to 1,500 base pairs, and most preferably 800 to 1,500 base pairs, which are highly homologous with the corresponding target sequence to enhance the probability of homologous recombination. The integrational elements may be any sequence that is homologous with the target sequence in the genome of the *Bacillus* cell. Furthermore, the integrational elements may be non-encoding or encoding nucleic acid sequences.

For autonomous replication, the vector may further comprise an origin of replication enabling the vector to replicate autonomously in the *Bacillus* cell in question. Examples of bacterial origins of replication are the origins of replication of plasmids pBR322, pUC19, pACYC177, and pACYC184 permitting replication in *E. coli*, and pUB110, pE194, pTA1060, and pAMß1 permitting replication in *Bacillus*. The origin of replication may be one having a mutation to make its function temperature-sensitive in the *Bacillus* cell (see, *e.g.*, Ehrlich, 1978, *Proceedings of the National Academy of Sciences USA* 75:1433).

The procedures used to ligate the elements described above to construct the recombinant expression vectors are well known to one skilled in the art (see, *e.g.*, Sambrook *et al.*, 1989, *supra*).

In the methods of the present invention, the *Bacillus* cell may be a wild-type *Bacillus* cell or a mutant thereof. Furthermore, the *Bacillus* cell may be an alkalophilic or a thermophilic *Bacillus*. In a preferred embodiment, the *Bacillus* cell is a *Bacillus alkalophilus* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus amyloliquefaciens* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus brevis* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus circulans* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus clausii* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus coagulans* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus firmus* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus lautus* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus lentus* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus licheniformis* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus megaterium* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus pumilus* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus stearothermophilus* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus subtilis* cell. In another preferred embodiment, the *Bacillus* cell is a *Bacillus thuringiensis* cell.

In the methods of the present invention, the *Bacillus* cell may be a recombinant cell comprising, for example, a nucleic acid sequence encoding a heterologous polypeptide.

The cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). The secreted polypeptide can be recovered directly from the medium.

The polypeptides may be detected using methods known in the art that are specific for the polypeptides. These detection methods may include use of specific antibodies, formation of an enzyme product, disappearance of an enzyme substrate, or SDS-PAGE.

For example, an enzyme assay may be used to determine the activity of the polypeptide. Procedures for determining enzyme activity are known in the art for many enzymes.

In the methods of the present invention, the *Bacillus* cell preferably produces at least about 25% more, more preferably at least about 50% more, more preferably at least about 75% more, more preferably at least about 100% more, even more preferably at least about 200% more, most preferably at least about 300% more, and even most preferably at least about 400% more polypeptide relative to a *Bacillus* cell containing only one of the promoter sequences of the tandem or "consensus" promoter operably linked to a nucleic acid sequence encoding the polypeptide when cultured under identical production conditions.

The resulting polypeptide may be isolated by methods known in the art. For example, the polypeptide may be isolated from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation. The isolated polypeptide may then be further purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing), differential solubility (*e.g.*, ammonium sulfate precipitation), or extraction (see, *e.g.*, *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

The present invention also relates to *Bacillus* cells comprising a tandem promoter in which each promoter sequence of the tandem promoter is operably linked to a single copy of a nucleic acid sequence encoding a polypeptide and alternatively also an mRNA processing/stabilizing sequence located downstream of the tandem promoter and upstream of the nucleic acid sequence encoding the polypeptide. In a preferred embodiment, the *Bacillus* cell is free of a selectable marker gene.

The present invention also relates to *Bacillus* cells comprising a nucleic acid construct which comprises (i) a "consensus" promoter operably linked to a single copy of a nucleic acid sequence encoding a polypeptide and (ii) an mRNA processing/stabilizing sequence located downstream of the "consensus" promoter and upstream of the nucleic acid sequence encoding the polypeptide.

The present invention also relates to *Bacillus* host cells comprising a nucleic acid construct comprising one or more copies of a "consensus" *amyQ* promoter operably linked to a single copy of a nucleic acid sequence encoding the polypeptide.

The present invention also relates to methods for obtaining a *Bacillus* host cell, comprising introducing into a *Bacillus* cell a nucleic acid construct comprising (i) a tandem promoter in which each promoter sequence of the tandem promoter is operably linked to a single copy of a nucleic acid sequence encoding a polypeptide and alternatively also (ii) an mRNA processing/stabilizing sequence located downstream of the tandem promoter and upstream of the nucleic acid sequence encoding the polypeptide.

The present invention also relates to methods for obtaining a *Bacillus* host cell, comprising introducing into a *Bacillus* cell a nucleic acid construct comprising (i) a "consensus" promoter operably linked to a single copy of a nucleic acid sequence encoding the polypeptide and (ii) an mRNA processing/stabilizing sequence located downstream of the "consensus" promoter and upstream of the nucleic acid sequence encoding the polypeptide.

The present invention also relates to methods for obtaining a *Bacillus* host cell, comprising introducing into a *Bacillus* cell a nucleic acid construct comprising one or more copies of a "consensus" *amyQ* promoter operably linked to a single copy of a nucleic acid sequence encoding the polypeptide.

The present invention also relates to methods for producing a selectable marker-free mutant of a *Bacillus* cell, comprising deleting a selectable marker gene of the *Bacillus* cell, wherein the *Bacillus* cell comprises a nucleic acid construct comprising (i) a tandem promoter in which each promoter sequence of the tandem promoter is operably linked to a single copy of a nucleic acid sequence encoding a polypeptide and alternatively also (ii) an mRNA processing/stabilizing sequence located downstream of the tandem promoter and upstream of the nucleic acid sequence encoding the polypeptide.

The present invention also relates to methods for producing a selectable marker-free mutant of a *Bacillus* cell, comprising deleting a selectable marker gene of the *Bacillus* cell, wherein the *Bacillus* cell comprises a nucleic acid construct comprising (i) a "consensus" promoter operably linked to a single copy of a nucleic acid sequence encoding the polypeptide and (ii) an mRNA processing/stabilizing sequence located downstream of the "consensus" promoter and upstream of the nucleic acid sequence encoding the polypeptide.

The present invention also relates to methods for producing a selectable marker-free mutant of a *Bacillus* cell, comprising deleting a selectable marker gene of the *Bacillus* cell, wherein the *Bacillus* cell comprises a nucleic acid construct comprising one or more copies of a "consensus" *amyQ* promoter operably linked to a single copy of a nucleic acid sequence encoding the polypeptide.

The present invention also relates to such selectable marker-free *Bacillus* mutants produced by the methods of the present invention.

The present invention also relates to an isolated nucleic acid sequence comprising the "consensus" *amyQ* promoter contained in SEQ ID NO. 26 or SEQ ID NO. 27.

The present invention is further described by the following examples which should not be construed as limiting the scope of the invention.

Examples

Bacterial strains

E. coli DH5α, *E. coli* JM101, and *Bacillus subtilis* PL1801 *spoIIE::Tn917* (*amyE, apr, npr*).

Primers and Oligos

All primers and oligos were synthesized on an Applied Biosystems Model 394 Synthesizer (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions.

Example 1: Construction of plasmid pDG268MCS

pDG268 (Antoniewski, *et al.*, 1990, *Journal of Bacteriology* 172: 86-93) was digested with *Tth*111I and *Eco*RI, and the digest was subjected to electrophoresis using a 0.7% agarose gel with 45 mM Tris-borate-1 mM EDTA (TBE). The largest plasmid fragment of approximately 6020 bp was excised from the gel and the DNA was recovered using the Qiaquick DNA purification kit (QIAGEN, Inc., Chatsworth, CA) according to the manufacturer's instructions. The recovered DNA was then ligated with the following

synthetic polylinker shown below to introduce unique *SfiI* and *BamHI* sites into the plasmid.

　　　　　　　　　SfiI 　　　*ApaI SmaI AatII HindIII* 　　*ClaI* 　　　　*BamHI*

NotI

5'-AATTGGCCTTAAGGGCCCGGGACGTCAAGCTTATCGATGCGGATCCGCGGCC GC-3'

3'-CCGGAATTCCCGGGCCCTGCAGTTCGAATAGCTACGCCTAGGCGCCGGCGC-5'

(SEQ ID NOs:1 and 2, respectively)

　　　E. coli DH5α was transformed with the ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. Plasmid DNA was purified according to Sambrook *et al.*, 1989, *supra*, and digested with *SfiI* and *NotI* to identify plasmids which contained these sites and by implication, the polylinker shown above (pDG268 does not contain these two restriction sites). Several plasmids were identified which contained both restriction sites and in addition were approximately 3.0 kb smaller than pDG268 as a result of replacing the *lacZ* gene of pDG268 with the synthetic polylinker. One such plasmid was chosen and designated pDG268MCS (MCS refers to multiple cloning site) (Figure 3).

Example 2: Construction of pDG268MCSΔ-Pr_{cryIIIA}/cryIIIAstab/SAV

　　　pDG268MCSΔ-Pr_{cryIIIA}/cryIIIAstab/SAV was constructed to delete the *SacI* site in pDG268MCS of Example 1 to facilitate the swapping of promoter fragments engineered to contain a *SacI* site at their 3' end. pDG268MCS-Pr_{cryIIIA}/cryIIIAstab/SAV (see Example 9 and Figure 12) was digested with *SnaBI* and *NruI* (both restriction sites flank the *SacI* site of the vector), ligated, and transformed into *E. coli* DH5α. Ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. Plasmid DNA was purified from several transformants and recovered as described in Example 1. The plasmid DNA was digested with *SacI* to identify plasmids which were deleted for the *SacI* site located in the vector sequence and thus cleaved only once due to the *SacI* site downstream of the *cryIIIA* promoter. Such a plasmid was identified and designated pDG268MCSΔ-Pr_{cryIIIA}/cryIIIAstab/SAV (Figure 4).

Example 3: Construction of pDG268MCSΔneo-Pr$_{CryIIIA}$/cryIIIAstab/SAV

The introduction of an expression cassette, contained in pDG268MCS or a pDG268MCSΔ derivative, into the chromosome of a *Bacillus* cell must be confirmed by PCR analysis since there is no way of distinguishing whether the introduction into the chromosome is a result of a double (desired) versus a single cross-over event. In order to alleviate this problem, pDG268MCSΔneo-Pr$_{CryIIIA}$/cryIIIAstab/SAV was constructed to contain an antibiotic resistance marker conferring resistance to neomycin, outside of the *amyE* "front" and *amyE* "back" regions of homology. A double cross-over results in Cm^r, Neo^s transformants whereas a single cross-over results in Cm^r, Neo^r transformants allowing the identification of the desired double cross-over events by screening for drug resistances rather than PCR.

pDG268MCSΔneo-Pr$_{CryIIIA}$/cryIIIAstab/SAV was constructed first by digesting pDG268MCSΔ-Pr$_{CryIIIA}$/cryIIIAstab/SAV of Example 2 with *Bal*I and treating with calf intestinal alkaline phosphatase. Plasmid pBEST501 (Itaya *et al.*, 1989, *Nucleic Acids Research* 17: 4410) was digested with *Pst*I and *Not*I, treated with T4 DNA polymerase I to generate blunt ends, and agarose gel purified as described in Example 1 to isolate a fragment harboring the neomycin resistance marker. The gel-purified fragment and *Bal*I-digested plasmid were ligated together and transformed into *E. coli* DH5α. Ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. The selected transformants were patched onto LB plates supplemented with 50 μg of neomycin per ml to identify neomycin resistant transformants. Plasmid DNA was purified from a few of the neomycin resistant transformants as described in Example 1 and digested with *Bgl*II (cuts twice due to the additional *Bgl*II site introduced with the neomycin resistance marker) yielding two fragments in the 4 kb range and with *Bam*HI (which is predicted to cut once downstream of the SAVINASE™ protease gene) yielding a fragment of approximately 8 kb. Such a plasmid was identified and designated pDG268MCSΔneo-Pr$_{cryIIIA}$/cryIIIAstab/SAV (Figure 5).

Example 4: Construction of pHP13ampMCS

pHP13-amp, a variant of pHP13 (Haima *et al.*, 1987, *Molecular and General Genetics* 209: 335-342), was constructed by digesting pUC9 with *Aat*II, blunting with Klenow fragment and deoxyribonucleotides, and then digesting with *Hind*III. The larger

2.2 kb fragment was gel-purified with a Qiaex kit (QIAGEN, Thousand Oaks, CA). pHP13 was digested with *HpaI* (which cuts within the erythromycin resistance gene), blunted, and then digested with *HindIII*. The larger 3.0 kb fragment released from pHP13 was then ligated to the 2.1 kb pUC9 fragment containing the pUC9 origin of replication and ampicillin resistance gene. The ligation mixture was transformed into *E. coli* DH5α. Ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. Plasmid DNA was purified from several transformants as described in Example 1. A plasmid designated pHP13amp was recovered from one of the transformants.

Plasmid pHP13amp was digested with *EcoRI* and *HindIII* and the pUC9 MCS was replaced with a new MCS created by annealing 100 pmol of the following polylinker in 50 mM NaCl, 10 mM Tris pH 7.5, and 1 mM EDTA, boiling for 5 minutes, and cooling slowly to room temperature over a 2 hour time period:

SfiI *ApaI* *SmaI*   *SacI*   *HindIII*   *NotI*   *NcoI*   *SalI*
                        (*Ecl*136II)

5'-AGCTAGGCCTTAAGGGCCCGGGACGTCGAGCTCAAGCTTGCGGCCGCCATGGTCGAC G-3'

3'-TCCGGAATTCCCGGGCCCTGCAGCTCGAGTTCGAACGCCGGCGGTACCAGCTGCTTA A-5' (SEQ ID NOs:3 and 4, respectively)

E. coli DH5α was transformed with the ligation mix and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. Plasmid DNA was purified from several transformants as described in Example 1 and digested with *NotI* and *SacI*. Plasmids which were cleaved with these enzymes contained the synthetic polylinker. One such plasmid was identified and designated pHP13amp-MCS (Figure 6). This plasmid was further verified by DNA sequencing through the polylinker region. DNA sequencing was performed with an Applied Biosystems Model 373A Automated DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions.

Example 5: Isolation of the SAVINASE™ serine protease gene

The gene, which encodes a *Bacillus* serine protease (SAVINASE™, Novo Nordisk A/S, Bagsværd, Denmark), hereinafter referred to as the SAVINASE™ gene, was PCR-

amplified using plasmid pSX222 (Figure 7, U.S. Patent No. 5,621,089) as template DNA and the following two primers (restriction sites are underlined):

ApaI   SacI

5'-CTCCGGGCCCATCTGAGCTCTATAAAAATGAGGAGGG-3' (SEQ ID NO. 5)

BamHI

5'-CCTCGGATCCATACACAAAAAAACGCT-3' (SEQ ID NO. 6)

The amplification reaction (100 µl) consisted of the following components: 50 ng of pSX222, 50 pmole of each primer, 1X *Taq* DNA polymerase Buffer (Boehringer Mannheim, Indianapolis, IN), 200 µM each of dATP, dTTP, dGTP, and dCTP, and 2.5 units of *Taq* DNA polymerase (Perkin-Elmer Corp., Branchburg, NJ). The amplification conditions were one cycle at 95°C for 3 minutes, 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes, and a final cycle at 72°C for 5 minutes.

The approximately 1230 bp PCR product was subcloned directly into the pCRII vector (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The sequence of the gene was verified by DNA sequencing as described in Example 4 using gene-specific primers. Once verified, the plasmid was digested with *Bam*HI, filled in with Klenow fragment, digested with *Apa*I, and the fragment harboring the SAVINASE™ gene was then ligated into the *Apa*I, *Eci*136II site of pHP13ampMCS. *E. coli* DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 µg of ampicillin per ml. A plasmid designated pHP13amp-SAV (Figure 8) was isolated from one of the transformants and verified by DNA sequencing as described in Example 4 using construct-specific primers. The *Bam*HI site was regenerated as a result of this ligation.

Example 6: Construction of an *amyL* promoter-SAVINASE™ gene expression cassette

The promoter of the *amyL* gene, which encodes a *Bacillus licheniformis* alpha-amylase (TERMAMYL™, Novo Nordisk A/S, Bagsværd, Denmark), described in U.S. Patent No. 5,698,415 was PCR-amplified using plasmid pPL1759 (U.S. Patent No. 5,698,415) as template DNA, the following two primers (restriction sites are underlined), and GENEAMP® XL PCR Kit (Perkin-Elmer Corp., Branchburg, NJ) according to the manufacturer's instructions:

SfiI

5'-CCA<u>GGCCTTAAGGGCC</u>GCATGCGTCCTTCTTTGTGCT-3' (SEQ ID NO. 7)

*Sac*I

5'-CCA<u>GAGCTC</u>CTTTCAATGTGTAACATATGA-3' (SEQ ID NO. 8)

The amplification reaction (100 μl) consisted of the following components: 50 ng of pPL1759, 50 pmole of each primer, 1X *Taq* polymerase buffer, 2.5 μM each of dATP, dTTP, dGTP, and dCTP, and 1.0 unit of *Taq* polymerase. The amplification conditions were one cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes; and a final cycle at 72°C for 5 minutes.

The approximately 620 bp PCR product containing the *amyL* promoter was ligated with *Ecl*136II-digested pUC118. *E. coli* DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pUC118-Pr$_{amyL}$ was purified from an ampicillin resistant transformant that appeared white in the presence of 5-bromo-4-chloro-3-inodlyl-â-D-galactopyranoside (Sigma, St. Louis, MO) and verified by DNA sequencing as described in Example 4, using M13/pUC sequencing primers and *amyL*-specific primers.

pHP13amp-SAV of Example 5 was digested with *Sfi*I and *Sac*I and purified by gel electrophoresis, as described in Example 1. The approximately 620 bp *Sfi*I-*Sac*I fragment of pUC118-Pr$_{amyL}$ bearing the *amyL* promoter was isolated by gel electrophoresis, as described in Example 1, and ligated to *Sfi*I/*Sac*I-digested pHP13amp-SAV. *E. coli* DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pHP13amp-Pr$_{amyL}$/SAV was purified from one of the ampicillin resistant transformants.

pHP13amp-Pr$_{amyL}$/SAV was digested with *Sfi*I and *Bam*HI and the approximately 1840 bp fragment bearing the Pr$_{amyL}$/SAV cassette was purified as described in Example 1. pDG268MCS was digested with *Sfi*I and *Bam*HI, purified as described in Example 1, and ligated with the Pr$_{amyL}$/SAV *Sfi*I/*Bam*HI fragment. *E. coli* DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCS-Pr$_{amyL}$/SAV (Figure 9) was purified from one of the ampicillin resistant transformants.

Example 7: Construction of an *amyQ* promoter-SAVINASE™ gene expression cassette

The promoter of the *amyQ* gene which encodes a *Bacillus amyloliquefaciens* alpha-amylase (BAN™, Novo Nordisk A/S, Bagsværd, Denmark) was PCR-amplified using plasmid pSX222 as template DNA and the following primers (restriction sites are underlined):

*Sfi*I

5'-TTT<u>GGCCTTAAGGGCC</u>TGCAATCGATTGTTTGAGAAAAGAAG-3' (SEQ ID NO. 9)

*Sac*I

5'-TTT<u>GAGCTC</u>CATTTTCTTATACAAATTATATTTTACATATCAG-3' (SEQ ID NO. 10)

The amplification reaction (100 µl) consisted of the following components: 50 ng of pSX222, 50 pmole of each primer, 1X *Taq* DNA polymerase buffer, 200 µM each of dATP, dTTP, dGTP, and dCTP, and 2.5 units of *Taq* DNA polymerase. The amplification conditions were one cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes; and a final cycle at 72°C for 5 minutes.

The approximately 185 bp PCR product was subcloned directly into the pCRII vector according to the manufacturer's instructions and verified by DNA sequencing as described in Example 4 yielding pCRII-Pr$_{amyQ}$. The approximately 185 bp *Sfi*I-*Sac*I fragment of pCRII-Pr$_{amyQ}$ bearing the *amyQ* promoter was isolated by gel electrophoresis, as described in Example 1, and ligated to *Sfi*I/*Sac*I-digested pHP13amp-SAV (Example 5). *E. coli* DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 µg of ampicillin per ml. A plasmid designated pHP13amp-Pr$_{amyQ}$/SAV was purified from one of the ampicillin resistant transformants.

pHP13amp-Pr$_{amyQ}$/SAV was digested with *Sfi*I and *Bam*HI, and the approximately 1400 bp fragment bearing the Pr$_{amyQ}$/SAV expression cassette was purified as described in Example 1. The *Sfi*I-*Bam*HI fragment was then ligated into *Sfi*I-*Bam*HI digested pDG268MCS (Example 6). *E. coli* DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 µg of

ampicillin per ml. A plasmid designated pDG268MCS-Pr_{amyQ}/SAV (Figure 10) was purified from one of the ampicillin resistant transformants.

Example 8: Construction of pUC18-Pr_{cryIIIA}/cryIIIAstab/cryIIIA

The promoter of the *cryIIIA* gene which encodes a *Bacillus thuringiensis* subsp. *tenebrionis* coleopteran crystal protein CryIIIA was PCR amplified from chromosomal DNA isolated according to Pitcher *et al.*, 1989, *Letters in Applied Microbiology* 8: 151-156 from the *Bacillus thuringiensis* subsp. *tenebrionis* strain NB125 described in WO 95/02695 using the following primers:

*Sma*I

5'-GAGACCCGGGAGCTTTCAGTGAAGTACGTG-3' (SEQ ID NO. 11)

5'-GGGGCGTTACAATTCAAAG-3' (SEQ ID NO. 12)

The amplification reaction (100 µl) consisted of the following components: 50 ng of NB125 chromosomal DNA, 50 pmole of each primer, 1X *Pfu* polymerase buffer (Stratagene Cloning Systems, La Jolla, CA), 200 µM each of dATP, dTTP, dGTP, and dCTP, and 1.0 unit of *Pfu* polymerase (Stratagene Cloning Systems, La Jolla, CA). The amplification conditions were one cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1.5 minutes; and a final cycle at 72°C for 5 minutes. The approximately 1000 bp PCR product was digested with *Sma*I and *Hind*III and ligated into the *Sma*I/*Hind*III site of pUC18. *E. coli* DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 µg of ampicillin per ml. A plasmid designated pUC18-Pr_{cryIIIA} was isolated from one of the ampicillin resistant transformants.

Plasmid pUC118-cryIIIA (WO 95/02695) was digested with *Hind*III and the approximately 3000 bp *Hind*III fragment harboring the *cryIIIA* gene and mRNA stabilizing sequence was gel-purified as described in Example 1. This fragment was ligated into the *Hind*III site of pUC18-Pr_{cryIIIA}. *E. coli* DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 µg of ampicillin per ml. A plasmid designated pUC18-Pr_{cryIIIA}/cryIIIAstab/cryIIIA (Figure 11) was isolated from one of the ampicillin resistant transformants. The correct orientation of the fragment was confirmed by digesting the plasmid with *Eco*RI.

Example 9: Construction of a *cryIIIA* promoter-*cryIIIA* mRNA stabilizer-SAVINASE™ gene expression cassette

The promoter and *cryIIIA* mRNA stabilizing sequence of the *cryIIIA* gene were PCR amplified using plasmid pUC18-Pr$_{cryIIIA}$/cryIIIAstab/cryIIIA of Example 8 as DNA template and the two primers (restriction sites are underlined) described below:

*Apa*I

5'-GGGCCCTCGAAACGTAAGATGAAACCT-3' (SEQ ID NO. 13)

*Sac*I

5'-GAGCTCCATAATACATAATTTTCAAACTG-3' (SEQ ID NO. 14)

The amplification reaction (100 μl) consisted of the following components: 50 ng of pUC18-Pr$_{cryIIIA}$/cryIIIAstab, 50 pmole of each primer, 1X *Taq* polymerase buffer, 200 μM each of dATP, dTTP, dGTP, and dCTP, and 1.0 unit of *Taq* polymerase. The amplification conditions were one cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes; and a final cycle at 72°C for 5 minutes.

The approximately 630 bp PCR product was cloned in the pCRII vector according to the manufacturer's instructions, yielding plasmid pCRII-Pr$_{cryIIIA}$/cryIIIAstab, which was verified by DNA sequencing as described in Example 4, using M13 sequencing primers and *cryIIIA*-specific primers.

The approximately 630 bp *Sfi*I-*Sac*I fragment of pCRII-Pr$_{cryIIIA}$/cryIIIAstab bearing the *cryIIIA* promoter (Pr$_{cryIIIA}$) with mRNA stabilizer sequence was isolated by gel electrophoresis, as described in Example 1, and ligated to *Sfi*I/*Sac*I-digested pHP13amp-SAV (Example 5). *E. coli* DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCS-Pr$_{cryIIIA}$/cryIIIAstab/SAV (Figure 12) was purified from one of the ampicillin resistant transformants.

Example 10: Construction of a *cryIIIA* promoter-SAVINASE™ gene expression cassette

pDG268Δneo-Pr$_{cryIIIA}$/SAV was constructed as follows. A fragment of approximately 1640 bp of pDG268MCSΔneo-Pr$_{cryIIIA}$/cryIIIAstab/SAV of Example 3 ranging from the *Dra*III site to 6 nucleotides downstream of the *cryIIIA* transcription start

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site was PCR-amplified using plasmid pDG268MCSΔneo-Pr$_{cryIIIA}$/cryIIIAstab/SAV as DNA template and the following two primers (restriction sites are underlined):

DraIII

5'-CAGCCAT<u>CACATTGTG</u>AAATC-3' (SEQ ID NO. 15)

SacI

5'-<u>GAGCTC</u>TATCTTTAATTAAGCTT-3' (SEQ ID NO. 16)

The amplification reaction (50 μl) consisted of the following components: 50 ng of pDG268MCSΔneo-Pr$_{cryIIIA}$/cryIIIAstab/SAV, 50 pmole of each primer, 1X Taq polymerase Buffer, 200 μM each of dATP, dTTP, dGTP, and dCTP, and 1.25 unit of Amplitaq® Gold DNA polymerase (Perkin-Elmer Corporation, Branchburg, NJ). The amplification conditions were one cycle at 95°C for 9 minutes; 30 cycles of 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes; and a final cycle at 72°C for 3 minutes. The PCR product was cloned directly into pCR2.1 using the TOPO-TA Cloning Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions yielding pCR2.1-DraIII/Pr$_{cryIIIA}$, which was verified by DNA sequencing as described in Example 4.

pDG268MCSΔneo- Pr$_{amyL}$/Pr$_{cryIIIA}$/cryIIIAstab/SAV of Example 12 was digested with DraIII and SacI to remove the Pr$_{amyL}$/Pr$_{cryIIIA}$/cryIIIAstab tandem promoter and part of the vector, and the approximately 6440 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. The approximately 1640 bp DraIII/SacI fragment of pCR2.1-DraIII/Pr$_{cryIIIA}$ was isolated and ligated with the DraIII/SacI-cut vector. E. coli DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCSΔneo-Pr$_{cryIIIA}$/SAV (Figure 13) was purified from one of the ampicillin resistant transformants.

Example 11: Construction of an *amyL* promoter-*cryIIIA* mRNA stabilizer-SAVINASE™ gene expression cassette

The *cryIIIA* mRNA stabilizer sequence was PCR-amplified using plasmid pUC18-Pr$_{cryIIIA}$/cryIIIAstab/cryIIIA of Example 8 as DNA template and the following two primers (*SacI* restriction sites are underlined):

5'-<u>GAGCTC</u>GAACTTGTTCATGTGAA-3' (SEQ ID NO. 17)

5'-<u>GAGCTC</u>ATAATACATAATTTTCA-3' (SEQ ID NO. 18)

The amplification reaction (100 µl) consisted of the following components: 50 ng of pUC18-Pr$_{cryIIIA}$/cryIIIAstab/cryIIIA, 50 pmole of each primer, 1X *Taq* DNA polymerase Buffer, 200 µM each of dATP, dTTP, dGTP, and dCTP, and 2.5 units of *Taq* DNA polymerase. The amplification conditions were one cycle at 95°C for 3 minutes, 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes, and a final cycle at 72°C for 5 minutes. The approximately 360 bp PCR product was subcloned directly into the pCRII vector according to the manufacturer's instructions and confirmed by DNA sequencing, as described in Example 4, using construct specific primers.

The resulting plasmid designated pCRII-cryIIIAstab was digested with *Sac*I and the fragment harboring the *cryIIIA* mRNA stabilizer sequence was purified by agarose gel electrophoresis as described in Example 1. Plasmid pHP13amp-Pr$_{amyL}$/SAV of Example 6 was digested with *Sac*I and treated with calf intestine alkaline phosphatase (CIP) to remove the 5' phosphate groups. The purified fragment containing the stabilizer sequence and the CIP-treated plasmid were then ligated together. *E. coli* DH5α cells were transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 µg of ampicillin per ml. Plasmid DNA was isolated from several transformants and digested with *Sac*I to identify those which contained the mRNA stabilizer sequence. Once these were identified, the orientation of the fragment was determined by digesting the plasmid DNA with *Xmn*I. A plasmid containing the *cryIIIA* stabilizing sequence in the correct orientation was identified and designated pHP13amp-Pr$_{amyL}$/cryIIIAstab/SAV.

pHP13amp-Pr$_{amyL}$/cryIIIAstab/SAV was digested with *Sfi*I and *Bam*HI and the fragment harboring the expression cassette was ligated into the *Sfi*I-*Bam*HI site of pDG268-MCS to produce pDG268MCS-Pr$_{amyL}$/cryIIIAstab/SAV (Figure 14).

Example 12: Construction of a tandem *amyL-cryIIIA* promoter-*cryIIIA* mRNA stabilizer-SAVINASE™ gene expression cassette

Plasmid pDG268MCSΔneo-Pr$_{cryIIIA}$/cryIIIAstab/SAV of Example 3 was digested with *Sfi*I, blunt-ended with T4 polymerase I, and then digested with *Dra*III. The digest was separated by agarose gel electrophoresis and the vector fragment of approximately 7010 bp was excised from the gel and the DNA extracted as described in Example 1.

pDG268MCS-Pr$_{amyL}$/SAV of Example 6 was digested with *Ecl*136II and *Dra*III. The digest was separated by agarose gel electrophoresis, the promoter fragment of approximately 2150 bp was excised from the gel, and the DNA was extracted as described in Example 1.

The purified DNAs were ligated together and transformed into *E. coli* DH5α. Ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCSΔneo-Pr$_{amyL}$/Pr$_{cryIIIA}$/cryIIIAstab/SAV (Figure 15) was isolated from one of the transformants and verified by digesting with *Nco*I followed by agarose gel electrophoresis.

Example 13: Construction of a tandem *amyL-cryIIIA* promoter-SAVINASE™ gene expression cassette

pDG268MCSΔneo-Pr$_{cryIIIA}$/SAV of Example 10 was digested with *Pac*I and *Dra*III, and the approximately 6450 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. The approximately 2230 bp *Pac*I-*Dra*III fragment of pDG268MCSΔneo-Pr$_{amyL}$/Pr$_{cryIIIA}$/cryIIIAstab/SAV of Example 12 bearing Pr$_{amyL}$ and Pr$_{cryIIIA}$ was isolated by gel electrophoresis, as described in Example 1, and ligated with the vector fragment. *E. coli* DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCSΔneo-Pr$_{amyL}$/Pr$_{cryIIIA}$/SAV (Figure 16) was purified from one of the ampicillin resistant transformants.

Example 14: Construction of an *amyQ* promoter-*cryIIIA* stabilizer-SAVINASE™ gene expression cassette

pDG268MCS-Pr$_{amyQ}$/SAV of Example 7 was digested with *Sfi*I and *Bam*HI and the approximately 1400 bp bearing the Pr$_{amyQ}$/SAV cassette was isolated by gel electrophoresis, as described in Example 1. pDG268MCSΔneo-Pr$_{cryIIIA}$/cryIIIAstab/SAV of Example 3 was digested with *Sfi*I and *Bam*HI and the approximately 6780 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. The purified fragments were ligated together, *E. coli* DH5α was transformed with the ligation mixture, and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCSΔneo-Pr$_{amyQ}$/SAV was purified from one of the

ampicillin resistant transformants and verified by digestion with *NcoI* followed by gel electrophoresis.

pDG268MCSΔneo-Pr$_{amyQ}$/cryIIIAstab/SAV was constructed first by digesting pDG268MCSΔneo-Pr$_{amyQ}$/SAV with *SacI* and treating with calf intestine alkaline phosphatase to remove the 5' phosphate groups. The approximately 360 bp *SacI* fragment of pDG268MCS-Pr$_{amyL}$/cryIIIAstab/SAV of Example 11 bearing the *cryIIIA* leader was gel-purified as described in Example 1 and ligated with *SacI*-cut, dephosphorylated pDG268MCSΔneo-Pr$_{amyQ}$/SAV.

E. coli DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. Plasmid DNA was isolated from several transformants and digested with *SacI* to identify those which contained the stabilizer sequence. Once these were identified, the orientation of the fragment was determined by digesting the plasmid DNA with *XmnI*. A plasmid containing the *cryIIIA* mRNA stabilizing sequence in the correct orientation was identified and designated pDG268MCSΔneo-Pr$_{amyQ}$/cryIIIAstab/SAV (Figure 17).

Example 15: Construction of a tandem *amyQ-cryIIIA* promoter-*cryIIIA* mRNA stabilizer-SAVINASE™ gene expression cassette

Plasmid pDG268MCS-Pr$_{cryIIIA}$/cryIIIAstab/SAV of Example 9 was digested with *SfiI*, blunt-ended with T4 polymerase I, and then digested with *DraIII*. The digest was subjected to agarose gel electrophoresis and the vector fragment of approximately 6360 bp was excised from the gel and the DNA extracted as described in Example 1.

Plasmid pDG268MCS-Pr$_{amyQ}$/SAV of Example 7 was digested with *SacI*, blunt-ended with T4 DNA polymerase I, digested with *DraIII*. The digest was subjected to agarose gel electrophoresis and the promoter fragment of approximately 1710 bp was excised from the gel and the DNA extracted as described in Example 1.

Purified DNAs were ligated together and transformed in *E. coli* DH5α cells. Ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCS-Pr$_{amyQ}$/Pr$_{cryIIIA}$/cryIIIAstab/SAV (Figure 18) was isolated from one of the ampicillin resistant transformants. The plasmid structure was verified by digesting with *SacI* followed by gel-electrophoresis.

Example 16: Construction of a tandem *amyQ-cryIIIA* promoter-SAVINASE™ gene expression cassette

pDG268MCSΔneo-Pr$_{cryIIIA}$/SAV of Example 10 was digested with *PacI* and *DraIII*, and the approximately 6450 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. The approximately 1790 bp *PacI-DraIII* fragment of pDG268MCS-Pr$_{amyQ}$/Pr$_{cryIIIA}$/cryIIIAstab/SAV (Example 15) bearing Pr$_{amyQ}$ and Pr$_{cryIIIA}$ was isolated by gel electrophoresis, as described in Example 1, and ligated with the vector fragment. *E. coli* DH5α was transformed with this ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCSΔneo-Pr$_{amyQ}$/Pr$_{cryIIIA}$/SAV (Figure 19) was purified from one of the ampicillin resistant transformants.

Example 17: Construction of a *cryIIIA* stabilizer-SAVINASE™ gene expression cassette

pDG268MCSΔneo-cryIIIAstab/SAV was constructed as follows: pDG268MCSΔneo-Pr$_{cryIIIA}$/cryIIIAstab/SAV of Example 3 was digested with *SfiI* and treated with T4 DNA polymerase to remove the 3' overhangs. The plasmid was then digested with *Asp*718. The digest was subjected to agarose gel electrophoresis and the *Asp*718/blunted *SfiI* vector fragment of approximately 7540 bp was excised from the gel and the DNA extracted as described in Example 1.

pDG268MCSΔneo-Pr$_{cryIIIA}$/cryIIIAstab/SAV was digested with *HindIII* and treated with T4 DNA polymerase and dNTPs to fill in the recessed 3' ends. The plasmid was then digested with *Asp*718. The digest was separated by agarose gel electrophoresis and the fragment of approximately 920 bp containing the truncated *cryIIIA* promoter (only the "-10" region) and the downstream cryIIIA stabilizing sequence was excised from the gel and the DNA extracted as described in Example 1.

Purified DNAs were ligated together and the ligation mixture was transformed into *E. coli* DH5α cells. Ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCSΔneo-cryIIIAstab/SAV (Figure 20) was purified from one of the transformants.

Example 18: Construction of a "consensus" *amyQ* promoter

The following two oligonucleotides were annealed together and extended with Klenow fragment to generate a 68 bp double stranded fragment containing mutations (*) in the -10 and -35 regions of the *amyQ* promoter (highlighted in bold letters):

<pre>
SOE   **   *
</pre>

5'-<u>GGAATAAAGGGGGG**TTGACA**TTATTTTACTGATATG**TATAAT**AT</u>-3' (SEQ ID NO. 19)

<pre>
                                                           SacI
</pre>

3'-AATAAAATGACTATACATATTATATTAAACATATTCTTTTACCTCGAG-5' (SEQ ID NO. 20)

A second double-stranded fragment comprising 137 bp of the upstream region of the *amyQ* promoter was generated by PCR using the following to primers:

<pre>
   SfiI
</pre>

5'-GGCCTTAGGGGCCTGCA-3' (SEQ ID NO. 22)

<pre>
        SOE
</pre>

5'-<u>TGTCAACCCCCCTTTATTCC</u>TT-3' (SEQ ID NO. 23)

Both double-stranded DNA fragments were then fused together by traditional SOE methods (SOE overlaps are underlined and labeled) to generate a mutated version of the *amyQ* promoter designated "consensus" amyQ. The primers used in the SOE reaction to obtain the full-length fragment were 5'-<u>GGCCTTAAGGGCCTGCA</u>-3'(SEQ ID NO. 22) and 5'-<u>GAGCTC</u>CATTTTCTTATACAAATTATAT-3' (SEQ ID NO. 24).

The SOE reaction (50 µl) consisted of the following components: 50 ng of the 68 bp SOE fragment and 50 ng of the 137 bp SOE fragment, 1X *Taq* polymerase buffer, 200 µM each of dATP, dTTP, dGTP, and dCTP, and 2.5 units of *Taq* DNA polymerase. The conditions were one cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes; after the third cycle 50 pmole of each of the two primers described above were added during the remaining 27 cycles to amplify the 185 bp promoter fragment. A final cycle was performed at 72°C for 5 minutes.

The approximately 185 bp PCR product was subcloned directly into the pCRII vector according to the manufacturer's instructions and verified by DNA sequencing as described in Example 4 yielding pCRII-Pr"consensus" amyQ, using forward and reverse M13 sequencing primers.

The sequence of the entire *amyQ* promoter including flanking restriction sites is shown in Figure 21 (SEQ ID NO. 25). The following mutations were introduced into the nucleic acid sequence containing the wild-type *amyQ* promoter (SEQ ID NO. 25) to generate the "consensus" *amyQ* promoter (SEQ ID NO. 26): T to A and T to C in the -35 region (with respect to the transcription start site) at positions 135 and 136, respectively, and an A to T change in the -10 region at position 156 of SEQ ID NO. 25. Also a T to A change was inadvertently made at position 116 approximately 20 base pairs upstream of the -35 region as shown in Figure 21 (SEQ ID NO. 27). This change apparently had no detrimental effect on promoter function since it is well removed from the critical -10 and -35 regions.

Example 19: Construction of a short "consensus" *amyQ* promoter-SAVINASE™ gene expression cassette

The short "consensus" *amyQ* promoter was PCR amplified from pCRII-Pr-consensus-amyQ (Example 18) using the following oligonucleotide primers:

5'- GGCCTTAAGGGCCTGCTGTCCAGACTGTCCGCT-3' (SEQ ID NO. 28)

5'- GAGCTCCATTTTCTTATACAAATTATAT-3' (SEQ ID NO. 24)

The amplification reaction (100 μl) consisted of the following components: 50 ng of pCRII-Pr-consensus-amyQ, 50 pmole of each primer, 1X *Taq* polymerase buffer, 200 μM each of dATP, dTTP, dGTP, and dCTP, and 1.0 unit of *Taq* polymerase. The amplification conditions were one cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 50°C for 1 minute, and 72°C for 1.5 minutes; and a final cycle at 72°C for 5 minutes.

The approximately 100 bp PCR product was cloned in the pCRII vector according to the manufacturer's instructions, yielding plasmid pCRII-Pr$_{short \ "consensus" \ amyQ}$, which was verified by DNA sequencing as described in Example 4, using forward and reverse M13 sequencing primers.

pCRII-Pr$_{short \ "consensus" \ amyQ}$ was digested with *Sfi*I and *Sac*I, and the approximately 100 bp fragment bearing the promoter was isolated by gel electrophoresis, as described in Example 1. pHP13amp-SAV of Example 5 was digested with *Sfi*I and *Sac*I, and the approximately 6430 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. The purified were ligated together, *Bacillus subtilis* PL1801 *spoIIE*::Tn917 was transformed with this ligation mixture, and chloramphenicol resistant transformants

were selected on TBAB plates supplemented with 5 μg of chloramphenicol per ml. A plasmid designated pHP13amp-Pr$_{short\ "consensus"\ amyQ}$/SAV was purified from one of the chloramphenicol resistant transformants.

pHP13amp-Pr$_{short\ "consensus"\ amyQ}$/SAV was digested with *Sfi*I and *Bam*HI, and the approximately 1330 bp fragment bearing the Pr$_{short\ "consensus"\ amyQ}$/SAV cassette was isolated by gel electrophoresis, as described in Example 1. pDG268MCS of Example 1 was digested with *Sfi*I and *Bam*HI, and the approximately 6040 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. The purified fragments were ligated together, *E. coli* DH5α was transformed with the ligation, and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCS-Pr$_{short\ "consensus"\ amyQ}$/SAV (Figure 22) was purified from one of the ampicillin-resistant transformants and verified by digestion with *Sfi*I and *Bam*HI followed by gel electrophoresis.

Example 20: Construction of an short "consensus" *amyQ* dimer promoter-SAVINASE™ gene expression cassette

pDG268MCS-Pr$_{short\ "consensus"\ amyQ}$/SAV of Example 19 was digested with *Sfi*I, treated with T4 DNA polymerase to generate blunt ends, and digested with *Dra*III. The approximately 5830 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. pDG268MCS-Pr$_{short\ "consensus"\ amyQ}$/SAV was also digested with *Ecl*136II and *Dra*III, and the approximately 1640 bp fragment bearing the Pr$_{short\ "consensus"\ amyQ}$/SAV was isolated by gel electrophoresis, as described in Example 1. The purified fragments were ligated together and transformed into *E. coli* DH5α cells. Ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCS-Pr$_{short\ "consensus"\ amyQ\ dimer}$/SAV (Figure 23) was purified from one of the ampicillin-resistant transformants and verified by DNA sequencing as described in Example 4 using construct-specific primers.

Example 21: Construction of a short "consensus" *amyQ* promoter-*cryIIIA* stabilizer-SAVINASE™ gene expression cassette

pDG268MCSΔ-Pr$_{cryIIIA}$/cryIIIAstab/SAV of Example 2 was digested with *Sfi*I and *Bam*HI, and the approximately 5390 bp vector fragment was isolated by gel

electrophoresis, as described in Example 1. pHP13amp-Pr$_{short}$ $_{"consensus"}$ $_{amyQ}$/SAV was digested with *SfiI* and *BamHI*, and the approximately 1330 bp fragment bearing the Pr$_{short}$ $_{"consensus"}$ $_{amyQ}$/SAV cassette was isolated by gel electrophoresis, as described in Example 1. The purified fragments were ligated together, *E. coli* DH5α was transformed with the ligation mixture, and ampicillin resistant transformants were selected on LB plates supplemented with 100 µg of ampicillin per ml. A plasmid designated pDG268MCSΔ-Pr$_{short}$ $_{"consensus"}$ $_{amyQ}$/SAV was purified from one of the ampicillin resistant transformants and verified by digestion with *NcoI* followed by gel electrophoresis.

pDG268MCSΔ-Pr$_{short}$ $_{"consensus"}$ $_{amyQ}$/SAV was digested with *SacI* and treated with calf intestine alkaline phosphatase to remove the 5′ phosphate groups. pDG268MCS-Pr$_{amyL}$/cryIIIAstab/SAV of Example 11 was digested with *SacI* and the approximately 350 bp fragment bearing the *cryIIIA* stabilizer was isolated by gel electrophoresis, as described in Example 1. pDG268MCSΔ-Pr$_{short}$ $_{"consensus"}$ $_{amyQ}$/SAV and the stabilizer fragment were ligated, *E. coli* DH5α was transformed with the ligation mixture, and ampicillin resistant transformants were selected on LB plates supplemented with 100 µg of ampicillin per ml. A plasmid designated pDG268MCSΔ-Pr$_{short}$ $_{"consensus"}$ $_{amyQ}$/cryIIIAstab/SAV (Figure 24) was purified from one of the ampicillin resistant transformants and verified by DNA sequencing as described in Example 4 using construct-specific primers.

Example 22: Construction of a tandem short "consensus" *amyQ-cryIIIA* promoter- *cryIIIA* mRNA stabilizer- SAVINASE™ gene expression cassette

pDG268MCSΔneo-Pr$_{cryIIIA}$/cryIIIAstab/SAV of Example 3 was digested with *SfiI* and *BamHI*. The approximately 6780 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. pDG268MCS- Pr$_{short}$ $_{"consensus"}$ $_{amyQ}$/SAV was digested with *SfiI* and *BamHI*. The approximately 1300 bp expression cassette fragment was isolated by gel electrophoresis, as described in Example 1. The purified fragments were ligated together and transformed into E. coli DH5α cells. Ampicillin resistant transformants were selected on LB plates supplemented with 100 µg of ampicilllin per ml. A plasmid designated pDG268MCSΔneo- Pr$_{short}$ $_{"consensus"}$ $_{amyQ}$/SAV was purified from one of the ampicillin resistant transformants and verified by digestion with *NcoI* followed by gel electrophoresis.

pDG268MCSΔneo-Pr$_{cryIIIA}$/cryIIIAstab/SAV of Example 3 was digested with *Sfi*I, treated with Klenow fragment to generate blunt ends, and digested with *Dra*III. The approximately 7060 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. pDG268MCSΔneo-Pr$_{short\ "consensus"\ amyQ}$/SAV was digested with *Ecl*136II and *Dra*III, and the approximately 1540 bp fragment bearing the short "consensus" *amyQ* promoter was isolated by gel electrophoresis, as described in Example 1. The purified fragments were ligated together and transformed into *E. coli* DH5α cells. Ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCSΔneo-Pr$_{short\ "consensus"\ amyQ}$/Pr$_{cryIIIA}$/cryIIIAstab/SAV (Figure 25) was purified from one of the ampicillin resistant transformants and verified by digestion with *Nco*I followed by gel electrophoresis.

Example 23: Construction of a short "consensus" *amyQ* trimer promoter-SAVINASE™ gene expression cassette

pDG268MCS-Pr$_{short\ "consensus"\ amyQ\ dimer}$/SAV of Example 20 was digested with *Sfi*I and *Bam*HI, and the approximately 1230 bp fragment bearing the Pr$_{short\ "consensus"\ amyQ\ dimer}$/SAV cassette was isolated by gel electrophoresis, as described in Example 1. pDG268MCSΔneo-Pr$_{cryIIIA}$/cryIIIAstab/SAV of Example 3 was digested with *Sfi*I and *Bam*HI, and the approximately 6750 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. The purified fragments were ligated together, *E. coli* DH5α was transformed with the ligation mixture, and ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCSΔneo-Pr$_{short\ "consensus"\ amyQ\ dimer}$/SAV was purified from one of the ampicillin resistant transformants and verified by digestion with *Nco*I followed by gel electrophoresis.

pDG268MCSΔneo-Pr$_{short\ "consensus"\ amyQ}$/SAV of Example 23 was digested with *Ecl*136II and *Bam*HI, and the approximately 6840 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. pDG268MCSΔneo-Pr$_{"short\ consensus\ amyQ"\ dimer}$/SAV was digested with *Sfi*I, treated with T4 DNA polymerase to generate blunt ends, and digested with *Bam*HI. The approximately 1230 bp fragment bearing the Pr$_{"short\ consensus\ amyQ"\ dimer}$/SAV cassette was isolated by gel electrophoresis, as described in Example 1. The purified fragments were ligated together, *Bacillus subtilis* PL1801 *spoIIE*::Tn*917* was

transformed with the ligation, and chloramphenicol resistant transformants were selected on TBAB plates supplemented with 5 μg of chloramphenicol per ml. A chloramphenicol resistant, neomycin sensitive integrant containing the Pr$_{short\ "consensus"\ amyQ\ trimer}$/SAV cassette was isolated.

Genomic DNA was isolated from this integrant using the QIAGEN bacterial DNA isolation protocol (QIAGEN, Inc., Santa Clarita, CA, and a fragment bearing the short "consensus" *amyQ* trimer promoter was amplified from the genomic DNA by PCR using the following oligonucleotide primers:

5'-CCGTCGCTATTGTAACCAGT-3' (SEQ ID NO. 29)

5'-CGACTTCCTCTTCCTCAGAG-3' (SEQ ID NO. 30)

The amplification reaction (100 μl) consisted of the following components: 50 ng of genomic DNA, 50 pmole of each primer, 1X *Taq* polymerase buffer, 200 μM each of dATP, dTTP, dGTP, and dCTP, and 1.0 unit of *Taq* polymerase. The amplification conditions were one cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes; and a final cycle at 72°C for 5 minutes.

The approximately 1078 bp PCR product was purified using the QIAquick PCR Purification Kit (QIAGEN Inc., Santa Clarita, CA), and the sequence of the short "consensus" *amyQ* trimer promoter was verified by DNA sequencing of the PCR product as described in Example 4 using construct-specific primers.

Example 24: Construction of a short "consensus" *amyQ* promoter-long *cryIIIA* mRNA stabilizer-SAVINASE™ gene expression cassette

pDG268MCS-Pr$_{cryIIIA}$/cryIIIAstab/SAV of Example 9 was digested with *Hind*III, treated with T4 DNA polymerase and dNTPs to generate blunt ends, and digested with *Asp*718. The approximately 913 bp fragment bearing the long *cryIIIA* stabilizer and part of the SAVINASE™ gene coding region was isolated by gel electrophoresis, as described in Example 1. pDG268MCSΔneo-Pr$_{short\ "consensus"\ amyQ}$/SAV of Example 23 was digested with *Ecl*136II and *Asp*718, and the approximately 7700 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. The purified fragments were ligated and transformed into *E. coli* DH5α cells. Ampicillin resistant transformants were selected on LB plates supplemented with 100 μg of ampicillin per ml. A plasmid designated pDG268MCSΔneo-Pr$_{short\ "consensus"\ amyQ}$/long cryIIIAstab/SAV (Figure 26) was purified from

one of the ampicillin resistant transformants and verified by DNA sequencing as described in Example 4 using construct-specific primers.

Example 25: Construction of a "short" *amyQ* dimer promoter-SAVINASE™ gene expression cassette

The "short" *amyQ* promoter was PCR amplified from pCRII-Pr$_{amyQ}$ (Example 7) using the following oligonucleotide primers:

5'-GGCCTTAAGGGCCTGCTGTCCAGACTGTCCGCT-3' (SEQ ID NO. 31)

5'-GAGCTCCATTTTCTTATACAAATTATAT-3' (SEQ ID NO. 24)

The amplification reaction (100 μl) consisted of the following components: 50 ng of pCRII-Pr$_{amyQ}$, 50 pmole of each primer, 1X *Taq* polymerase buffer, 200 μM each of dATP, dTTP, dGTP, and dCTP, and 1.0 unit of *Taq* polymerase. The amplification conditions were one cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes; and a final cycle at 72°C for 5 minutes.

The approximately 100 bp PCR product was cloned in the pCRII vector according to the manufacturer's instructions, yielding plasmid pCRII-Pr$_{"short" amyQ}$, which was verified by DNA sequencing as described in Example 4, using forward and reverse M13 sequencing primers. Next, this plasmid was digested with *Sfi*I and *Sac*I and the approximately 100 bp promoter fragment was isolated by gel electrophoresis, as described in Example 1. pHP13amp-Pr$_{amyL}$/SAV from Example 6 was digested with *Sfi*I and *Sac*I and the approximately 6400 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. These two fragments were then ligated together and transformed into *Bacillus subtilis* 168Δ4 cells (WO 98/22598). Chloramphenicol resistant transformants were selected on TBAB plates supplemented with 5 μg of chloramphenicol per ml and overlayed with 2% dried milk in TBAB agar in order to identify SAVINASE expressing clones by halo formation around transformant colonies. A plasmid designated pHP13amp- Pr$_{"short" amyQ}$/SAV was purified from one of the chloramphenicol resistant, halo-forming transformants and verified by digestion with *Pvu*II followed by gel electrophoresis. Next, this plasmid was digested with *Sfi*I and *Bam*HI and the approximately 1300 bp expression cassette fragment was isolated by gel electrophoresis, as described in Example 1. pDG268MCS from Example 4 was digested with *Sfi*I and *Bam*HI. The two fragments were then ligated together and transformed into *E. coli* DH5α cells. Ampicillin resistant

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transformants were selected on LB plates supplemented with 100 µg of ampicilllin per ml. A plasmid designated pDG268MCS-Pr$_{\text{"short" amyQ}}$/SAV (Figure 27) was purified from one of the ampicillin resistant transformants and verified by digestion with *NcoI* followed by gel electrophoresis.

pDG268MCS-Pr$_{\text{"short" amyQ}}$/SAV was digested with *SfiI* and *BamHI*, and the approximately 1330 bp fragment bearing the Pr$_{\text{"short" amyQ}}$/SAV expression cassette was isolated by gel electrophoresis, as described in Example 1. pDG268MCSΔneo-Pr$_{\text{cryIIIA}}$/cryIIIAstab/SAV of Example 3 was digested with *SfiI* and *BamHI*, and the approximately 6750 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. The purified fragments were ligated together and transformed into *E. coli* DH5α cells. Ampicillin resistant transformants were selected on LB plates supplemented with 100 µg of ampicillin per ml. A plasmid designated pDG268Δneo-Pr$_{\text{"short" amyQ}}$/SAV was purified from one of the ampicillin resistant transformants and verified by digestion with *NcoI* followed by gel electrophoresis.

pDG268Δneo-Pr$_{\text{"short" amyQ}}$/SAV was digested with *SfiI*, treated with T4 DNA polymerase to generate blunt ends, and digested with *DraIII*. The approximately 6530 bp vector fragment was isolated by gel electrophoresis, as described in Example 1. pDG268Δneo-Pr$_{\text{"short" amyQ}}$/SAV was also digested with *DraIII* and *Ecl136II*, and the approximately 1640 bp fragment bearing the "short" *amyQ* promoter was isolated by gel electrophoresis, as described in Example 1. The purified fragments were ligated, *E. coli* DH5α was transformed with the ligation mixture, and ampicillin resistant transformants were selected on LB plates supplemented with 100 µg of ampicillin per ml. A plasmid designated pDG268Δneo-Pr$_{\text{"short" amyQ dimer}}$/SAV (Figure 28) was purified from one of the ampicillin resistant transformants and verified by DNA sequencing as described in Example 4 using construct-specific primers.

Example 26: Construction of a "short" *amyQ* dimer promoter-*cryIIIA* mRNA stabilizer-SAVINASE™ gene expression cassette

pDG268Δneo-Pr$_{\text{"short" amyQ dimer}}$/SAV of Example 25 was digested with *SacI* and treated with calf intestine alkaline phosphatase to remove the 5' phosphate groups. pDG268MCS-Pr$_{\text{amyL}}$/cryIIIAstab/SAV of Example 11 was digested with *SacI* and the approximately 350 bp fragment bearing the *cryIIIA* stabilizer was isolated by gel

electrophoresis, as described in Example 1. The *cryIIIA* stabilizer fragment was ligated to *Sac*I-cut pDG268Δneo-Pr·$_{short}$ $_{amyQ}$ $_{dimer}$/SAV. *E. coli* DH5α was transformed with the ligation mixture and ampicillin resistant transformants were selected on LB plates supplemented with 100 µg of ampicillin per ml. A plasmid designated pDG268Δneo-Pr·$_{short}$ $_{amyQ}$ $_{dimer}$/cryIIIAstab/SAV (Figure 29) was purified from one of the ampicillin resistant transformants and verified by DNA sequencing as described in Example 4 using construct-specific primers.

Example 27: Copy number of SAVINASE™ gene expression cassettes in *Bacillus subtilis* PL1801 *spoIIE*::Tn*917* integrants

The SAVINASE™ gene expression cassettes were each integrated into the *amyE* locus of *Bacillus subtilis* strain PL1801 *spoIIE*::Tn*917*. Specifically, pDG268MCS derivatives containing the expression cassettes were digested with *Sca*I to linearize the plasmids. One microgram of linearized plasmid DNA was used to transform competent *Bacillus subtilis* PL1801 *spoIIE*::Tn*917* to chloramphenicol resistance.

All transformants should contain a single copy of the expression cassette in the *amyE* locus as a result of a double cross-over event. This was confirmed by DNA dot blot analyses on chromosomal DNA to show the absence of the ampicillin-resistance gene (in integrants derived from pDG268MCS-or pDG268MCSΔ-based plasmids) or by neomycin sensitivity (in integrants derived from pDG268MCSΔneo-based plasmids).

Genomic DNA was isolated from *Bacillus subtilis* integrants using the QIAGEN bacterial DNA isolation protocol. For dot blot analysis, approximately 3 µg of genomic DNA were denatured with 400 mM NaOH-10 mM EDTA in a total volume of 10 µl, incubating at room temperature for 10 minutes. One µl of denatured DNA was spotted on a Boehringer-Mannheim positively-charged nylon membrane (Boehringer-Mannheim Corporation, Indianapolis, IN) and fixed by UV-crosslinking with a UV STRATALINKER™ 2400 (Stratagene Cloning Systems, La Jolla, CA). The membrane was probed with a DNA probe specific to the ampicillin-resistance gene *bla*, using the GENIUS™ System, Version 2.0 (Boehringer-Mannheim Corporation, Indianapolis, IN). The blot was developed according to the GENIUS protocol using ATTOPHOS® detection reagent (Amersham International, Little Chalfont, UK). The probe was detected using the STORM™ 860 optical scanner and ImageQuant software (Molecular Dynamics, Sunnyvale,

CA). Inability of the probe to bind to genomic DNA indicated absence of the *bla* gene, confirming insertion of the plasmid by double cross-over.

The *bla* probe was created by labeling a PCR fragment with digoxigenin-11-dUTP using the Boehringer-Mannheim PCR DIG Probe Synthesis Kit (Boehringer-Mannheim Corporation, Indianapolis, IN). The PCR was performed using Amplitaq® Gold DNA polymerase and a Robocycler™ 40 thermal cycler (Stratagene Cloning Systems, La Jolla, CA) with the following temperature profile: 9 minutes at 95°C; 30 cycles of 1 minute each at 95°C, 55°C, and 72°C; and 3 minutes at 72°C. The probe was amplified from pUC118 using the following oligonucleotide primers:

5'-CTATGTGGCGCGGTATTATC-3' (SEQ ID NO. 32)

5'-TTCATCCATAGTTGCCTGAC-3' (SEQ ID NO. 33)

Once the transformants were confirmed to contain a single copy of the SAVINASE™ expression cassette, single-copy integrants for each expression cassette were analyzed by shake flask analysis.

Example 28: SAVINASE™ expression in *Bacillus subtilis* PL1801 *spoIIE*::Tn917

The single-copy integrants of each expression cassette described in Example 19 were grown to mid-log phase in 1 ml of LB broth at 37°C. Then 100 µl of each mid-log phase culture was inoculated into 50 ml of PS-1 medium composed of 10% sucrose, 4% soybean flour, 1% $Na_2PO_4 \cdot 12H_2O$, 0.5% $CaCO_3$, and 0.01% pluronic acid. The cultures were shaken vigorously at 37°C for 5 days. One ml aliquots were removed on days 3, 4, and 5, centrifuged at 12,000 X g for 2 minutes, and 0.5 ml of each supernatant was frozen at -20°C until all samples were taken. The frozen samples were then thawed and assayed for SAVINASE™ activity using the following protocol to determine relative yields.

The assay for SAVINASE™ activity was performed with casein fluorescein isothiocyanate as substrate. The casein fluorescein isothiocyanate stock solution was prepared according to Twining, 1984, *Analytical Biochemistry* 143: 30-34 and contained 0.5 mg of casein fluorescein isothiocyanate per 100 ml of 50 mM Tris-Cl pH 7.2 buffer. The assay reaction was initiated by the addition of 40 µl of the stock solution mixed 1:1 v/v with 0.25 M borate pH 9.0 buffer to 10 µl of the enzyme sample diluted in 0.25 M borate pH 9.0 buffer as appropriate. The reaction was incubated for 10 minutes at 37°C and then quenched by adding 150 µl of 5% trichloroacetic acid. The quenched reaction was placed

in the cold room for 10 minutes and then centrifuged at top speed for 2 minutes. A 10 μl aliquot of the supernatant was transferred to a test tube containing 2 ml of 0.5 M borate pH 9.0 buffer and mixed well. A 200 μl aliquot of this solution was transferred to a black "U" bottom 96 well plate (Dynatech Laboratories, Inc., Chantilly, VA) and the fluorescence was measured using a Fluorolite 1000 fluorimeter (Dynatech Laboratories, Inc., Chantilly, VA) using channel 3 at reference setting 1176 and a lamp voltage at 4.1V. SAVINASE™ activity was calculated by reference to a standard curve generated with a SAVINASE™ standard (Novo Nordisk A/S, Bagsværd, Denmark) in the range of 1.8-9.0 NPU (Novo Protease Unit) per ml. The activity of the standard is determined according to Novo Analytical Method AF 220/1-GB available upon request from Novo Nordisk A/S, Bagsværd, Denmark.

The results are shown in Table 1. The *amyQ*, *amyL*, and *cryIIIA* promoters (without the stabilizer sequence) were all about equal in strength based on the SAVINASE™ assay results. When the *cryIIIA* mRNA stabilizer sequence was placed downstream of these promoters their activities increased substantially (at least a 1.7-fold increase in expression levels). However, when the *cryIIIA* mRNA stabilizer sequence was included downstream of the tandem promoters, the activities were more than four-fold higher than the single promoters by themselves and more than 2-fold higher than the single promoters with the *cryIIIA* mRNA stabilizer sequence included. When the *cryIIIA* mRNA stabilizer sequence was used without a promoter, SAVINASE™ expression was very low, indicating that the enhancing effect of the stabilizer sequence was not due to promoter activity within the stabilizer sequence. Therefore, by placing a promoter such as the *amyQ* or *amyL* promoter upstream of the *cryIIIA* promoter and its mRNA stabilizing sequence, a tandem promoter can be created which is far superior activity to any single promoter by itself.

The results also indicated it was possible to obtain higher levels of gene expression by modifying a promoter such as the *amyQ* promoter as described herein to make it a stronger promoter, *i.e.*, the "consensus" *amyQ* and "short consensus" *amyQ* promoters. Both of these promoters were approximately 4-fold stronger than the wild-type *amyQ* promoter from which they were derived. In addition, higher levels of expression could be obtained by placing the *cryIIIA* long mRNA stabilizer sequence downstream of the "short consensus" promoter. This particular combination resulted in saturating levels of mRNA

and maximum levels of SAVINASE™ expression, levels comparable to a fully amplified strain (a 6-fold increase over the wild-type amyQ promoter alone).

In addition, the results showed that higher levels of expression could be obtained by assembling more than one copy of a single promoter in a "head-to-tail" fashion. This is clearly illustrated by comparing the levels of expression obtained from the single short "consensus" amyQ promoter, a dimer short "consensus" amyQ promoter, and a trimer short "consensus" amyQ promoter. SAVINASE™ expression levels were 1.5-fold and 1.8-fold higher, respectively, for the latter two in comparison to the monomer promoter.

The overall results demonstrated the ability to increase transcription of a single-copy gene using the following approaches: The creation of tandem promoters by placing promoters such as amyQ and amyL upstream of the cryIIIA promoter and its mRNA stabilizing sequence, the creation of a "consensus" amyQ promoter with the cryIIIA mRNA stabilizing sequence, and the creation of tandem copies of a single promoter such as the short "consensus" amyQ dimer and trimer promoters. All of these approaches lead to significantly higher levels of gene expression when compared to the levels obtained using single promoters such as amyQ and amyL.

Table 1

Expression cassette	Relative SAVINASE™ activity
Pr_{amyL}/SAV	90%
Pr_{amyQ}/SAV	100%
$Pr_{short\ amyQ}$/SAV	100%
$Pr_{cryIIIA}$/cryIIIAstab/SAV	350%
$Pr_{cryIIIA}$/SAV	100%
Pr_{amyL}/cryIIIAstab/SAV	230%
Pr_{amyQ}/cryIIIAstab/SAV	170%
Pr_{amyL}/$Pr_{cryIIIA}$/SAV	90%
Pr_{amyQ}/$Pr_{cryIIIA}$/SAV	80%
Pr_{amyL}/$Pr_{cryIIIA}$/cryIIIAstab/SAV	570%
Pr_{amyQ}/$Pr_{cryIIIA}$/cryIIIAstab/SAV	540%
$Pr_{"short"\ amyQ\ dimer}$/SAV	210%
$Pr_{"short"\ amyQ\ dimer}$/cryIIIAstab/SAV	310%
$Pr_{short\ "consensus"\ amyQ}$/SAV	370%
$Pr_{short\ "consensus"\ amyQ}$/cryIIIAstab/SAV	620%
$Pr_{short\ "consensus"\ amyQ}$/long cryIIIAstab/SAV	550%
$Pr_{short\ "consensus"\ amyQ}$/$Pr_{cryIIIA}$/cryIIIAstab/SAV	700%

$Pr_{short\ "consensus"\ amyQ\ dimer}$/SAV   570%

$Pr_{short\ "consensus"\ amyQ\ trimer}$/SAV   670%

Long cryIIIAstab/SAV   3%